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RELEASE OF A GUINEA PIG EPIDERMAL CELLULAR COMPONENT BY ULTRAVIOLET LIGHT*

OWEN BLACK, JR. PH.D.† AND J. HILL ANGLIN, JR. PH.D.

ABSTRACT

The 25,000 × g supernatants obtained from UV irradiated epidermis homogenized in 0.3 M sucrose are more opalescent than are similar preparations from non-irradiated skin. These fractions from UV irradiated guinea pig epidermis contain more cholesterol (as free cholesterol) and protein than do similar preparations from non-irradiated skins. The protein associated with this material had an electrophoretic mobility similar to the α-globulins and was immunologically identical to a serum α-globulin. Electron microscopic examination showed the material had a spheroidal, globular appearance lacking definite structure. The material released by UV was also found to be immunologically, electrophoretically and chromatographically similar to material released by incubation of non-irradiated epidermal preparations with phospholipase A.

The supernatant fraction of epidermal homogenates prepared in 0.3 M sucrose from ultraviolet light (UV) irradiated guinea pig skins were consistently observed to be more turbid or opalescent than were the same fractions prepared from non-irradiated skins. Preliminary experiments verified this observation and disclosed that this material was of a large size, contained protein, and was sensitive to osmotic pressure changes. Some of the physical and biochemical properties of this material have been determined. This material has also been compared to similar material recovered from epidermis following incubation with phospholipase A (phosphatide acylhydrolase EC 3.1.1.4).

MATERIALS AND METHODS

Animals. White and albino male guinea pigs, weighing from 350 to 600 gm, were used for this study. The hair was shaved from the dorsal surface of these animals just prior to irradiation.

Irradiation procedure. Experiments involved in the initial isolation of the opalescent material utilized animals irradiated with a bank of four Westinghouse Sun Lamps (5000 erg/cm²/sec) as described previously (1). The irradiation period was three hours, resulting in 12 MED (guinea pig minimal erythema dose).

Most of the data reported in this study employed a Burdick mercury vapor lamp (QA 450N, 1.5×10^5 ergs/cm²/sec, Burdick Corporation, Milton, Wisconsin). The irradiation period with this source was 12 minutes (12 MED). Both methods appeared to produce equivalent effects.

Preparation of epidermal supernatant. The animals were killed by cervical luxation at specified times following irradiation. The pelts were removed and scraped with a razor blade carefully to minimize contamination of the epidermis with dermis (2). This operation took less than 10 minutes. The collected epidermis was weighed and homogenized with glass tissue grinders in cold 0.3 M sucrose (10 ml/gm wet tissue weight). The resulting homogenates were centrifuged for 10 minutes at 700 × g. This supernatant was then centrifuged at 25,000 × g for 15 minutes to obtain the supernatant used for further study.

The sucrose solutions utilized in these experiments contained 0.02% ethylmercuithioxalicylate (merthiolate) to retard microbial growth. This concentration of merthiolate was found not to affect the experimental results.

Gel filtration. Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, New Jersey) was equilibrated with 0.3 M sucrose at 2°C. Chromatographic columns were prepared with this material in small columns (2.4 × 36 cm) for single animal experiments or larger columns (5 × 73 cm) for multiple animal experiments. All column chromatography was performed in a cold room at 2°C. Following application of the sample, in not more than 5% of the resin bed volume, the columns were eluted with 0.3 M sucrose solution. Fractions of the

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* From the Departments of Biochemistry, Molecular Biology and Dermatology, University of Oklahoma Medical Center, 800 Northeast 13th Street, Oklahoma City, Oklahoma 73104. Reprint requests to Doctor Anglin.

† Present address: Department of Pharmacology, Baylor University Medical College, Texas Medical Center, Houston, Texas 77025.

eluate were collected in a drop counting fraction collector and assayed for protein by the method of Lowry, *et al.* (3). The opalescent fractions were pooled, put in dialysis tubing and reduced in volume with polyethylene glycol flakes.

Conditions affecting the amount of complex released. The contribution of proteins derived from infused plasma to the observed opalescence was evaluated in the following manner. Shaved guinea pig skin was excised and placed in a petri dish with 10 ml Eagle's Minimum Essential Media (Grand Island Biochemicals Co., Grand Island, New York). The skin was irradiated with the Burdick lamp, incubated four hours at 37°C, then processed in the usual manner. Other pelts were excised, irradiated *in vitro*, and processed immediately following irradiation.

Thermal injury has been shown by Sevitt (4) to produce vascular changes and a biphasic edema response *in vivo*. Thermal injury, *in vitro*, was utilized to determine whether an injury, other than UV, would release opalescent material in the same way UV irradiation *in vitro* did. For this test the epidermal surface of an excised pelt was placed in contact with a metal beaker containing water at 59°C for 10 minutes (4). The pelt was processed immediately.

Epidermal preparations from non-irradiated skins and skins irradiated *in vivo* were homogenized in 0.9% sodium chloride to test whether homogenization in solutions of lower osmotic pressure would affect the amount of protein recovered in the opalescent fractions.

Characterization of the opalescent material. a) *Electrophoresis.* Aliquots of opalescent material from epidermal preparations from non-irradiated skins and those from skins irradiated *in vitro* were examined by electrophoresis on Sepraphore III (Gelman Instrument Co., Ann Arbor, Michigan) cellulose acetate strips (2.5 × 17 cm). Electrophoresis was performed in cold 0.05 ionic strength tris barbital-sodium barbital buffer, pH 8.8 (Gelman HR Buffer), at one milliampere per strip for one hour. Protein in the strips was visualized with Ponceau S stain.

b) *Immunology.* Immunological techniques were employed to determine the number of proteins in the opalescent material and to investigate their relationship to serum proteins. Antiserum against the opalescent material was prepared by subcutaneous injection of the material mixed with Freund's complete adjuvant into a rabbit (5). Within two weeks the rabbit serum contained a useful titre of antibodies. Commercially available rabbit anti-guinea pig serum antiserum (Mann Research, Inc., New York, New York) was also uti'ized. Double diffusion experiments were performed (6) in 1% agarose plates (Mann Ago-Immunodiffusion Plates) incubated at room temperature. Immunoelectrophoretic techniques (7) utilizing both cellulose acetate strips and agar plates were employed to determine the specific serum protein association.

c) *Electron microscopy.* An aliquot of the opalescent material isolated from epidermis irradiated *in vivo* was applied to formovar-coated grids, air dried, and stained with a solution of 1% phosphotungstic acid, pH 7.2, for examination by electron microscopy.

d) *Chemical analysis.* Opalescent material from both non-irradiated epidermis and epidermis irradiated *in vivo* were extracted with chloroform-methanol by the method of Folch, *et al.* (8). Assays for cholesterol were performed on these extracts by the method of Zak (9). Protein in the opalescent material was assayed prior to extraction with chloroform-methanol.

e) *Thin layer chromatography.* Thin layer chromatography was used for qualitative comparison of neutral lipids and phospholipids in opalescent material from non-irradiated samples and material irradiated *in vivo*. The thin layer plates were of Silica Gel G, 500 microns thick, activated at 100°C for one hour. The plates were developed in chloroform-methanol water (65/25/4) for phospholipids (10), or petroleum ether-diethyl ether-acetic acid (90/10/1) for neutral lipids (11). Phospholipids were detected by spraying with Zinzadze reagent (12). Neutral lipids were visualized either by spraying with an aqueous 50% sulfuric acid solution (containing 1% dichromate) and heating, or by placing the plates in a closed container with iodine vapor. The lipids were characterized by comparing their mobility with that of authentic lipids on the same plate.

f) *Solubilization with phospholipase A.* Homogenates of nonirradiated epidermis were allowed to equilibrate to room temperature before adding 0.34 mg phospholipase A per gm wet tissue. The mixture was incubated at room temperature with gentle stirring for one hour. The usual centrifugation and gel filtration procedures were followed.

RESULTS

Figure 1 shows the elution pattern of protein contained in the 25,000 × g supernatent fraction obtained from epidermis collected from four irradiated and four nonirradiated guinea pig skins. This graph and all subsequent graphs of protein profiles are shown with the ordinates designated as relative absorbance, i.e., the absorbance derived from the Lowry protein assay measured in a Cary Model 14 spectrophotometer. The abcissa is designated as tube number and is the sequence of fractions from the fraction collector. Figure 1 shows that the protein associated with the opalescent fractions (in both irradiated and non-irradiated preparations) is excluded by the gel and eluted in the void volume (shown by the small arrow), implying a molecular weight greater than 200,000. The area under the curve indicates that the irradiated sample has about twice as much protein as the control sample.

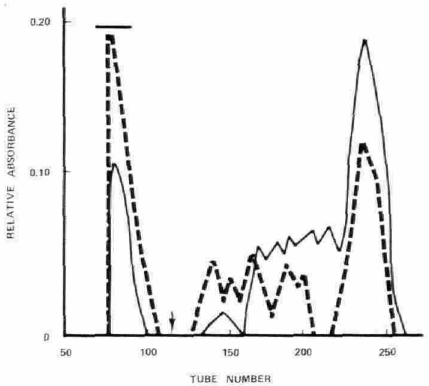

FIG. 1. Graph of protein in fractions from Sephadex G-200 columns comparing ----- a skin preparation irradiated *in vivo* (8.11 gm wet tissue weight, pooled from four animals) with ——— a non-irradiated preparation (9.37 gm wet tissue weight pooled from four animals). The material eluted prior to the arrow (which denotes void volume) was excluded by the gel. The bar above the initial peak denotes opalescent fractions.

Although opalescence was noted in non-irradiated preparations, the opalescence and protein content was invariably greater in equivalent preparations of irradiated epidermis.[1]

Conditions affecting the amount of complex released. The protein elution patterns from experiments designed to investigate the conditions affecting the amount of protein in opalescent material are shown in Figure 2. These experiments were repeated and the patterns in Figure 2 are typical of the several experiments performed. Figure 2A shows a graph of protein in opalescent fractions from representative Sephadex columns of (a) epidermis from a pelt irradiated *in vitro* and processed after a four hour delay, (b) epidermis from a pelt irradiated *in vivo* and processed after a four hour delay, (c) epidermis from a pelt irradiated *in vitro* and processed immediately. It can be seen that there is essentially no difference in the amount of protein in opalescent fractions whether the skin was irradiated *in vitro* or *in vivo*. It appears that the amount of protein is greater if the tissue is processed immediately rather than after a four hour delay. Figure 2B shows that thermal injury of epidermis, *in vitro*, does not increase the amount of protein in opalescent fractions when compared to non-treated control preparations. Figure 2C shows that homogenization of irradiated or non-irradiated epidermal tissue in 0.9% sodium chloride results in larger amounts of protein in opalescent fractions when compared to homogenates prepared in sucrose as shown in Figures 2A and 2B.

Characterization of the opalescent material. a) *Electrophoresis.* Electrophoresis of opalescent material from irradiated and non-irradiated epidermal preparations indicated that they contained only a single protein band which migrated with the α-globulins.

b) *Immunology.* The immunological investigations indicated that there was a single band of identity between the irradiated and non-irradiated samples, both to the anti-opalescent ma-

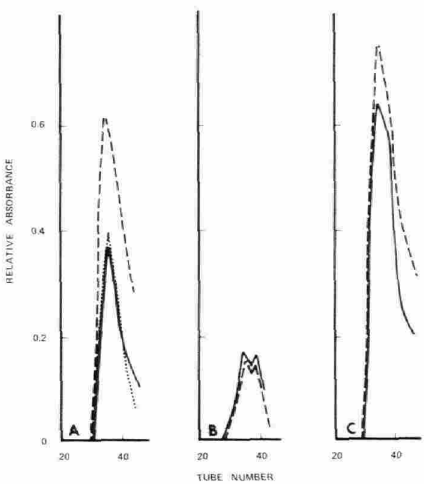

FIG. 2. A — Graphs of protein in opalescent fractions from Sephadex columns. —— Tissue (3.21 gm wet weight) from pelt irradiated *in vitro* and processed after four hour delay. ----- Tissue (2.82 gm wet weight) from pelt irradiated *in vivo* and processed after four hour delay. —— —— Tissue (2.98 gm wet weight) from pelt irradiated *in vitro* and processed immediately. B — Graphs of protein in opalescent fractions from Sephadex columns of – – – heat-treated (3.80 gm wet weight) and —— non-treated samples (2.32 gm wet weight) C — Graph of protein in opalescent fractions from Sephadex columns of – – – irradiated saline-homogenized (2.90 gm wet weight) and —— non-irradiated saline-homogenized (3.12 gm wet weight) samples.

[1] The amount of opalescence is a subjective observation and is considered to result from the presence of particles having an index of refraction different from the sucrose solution.

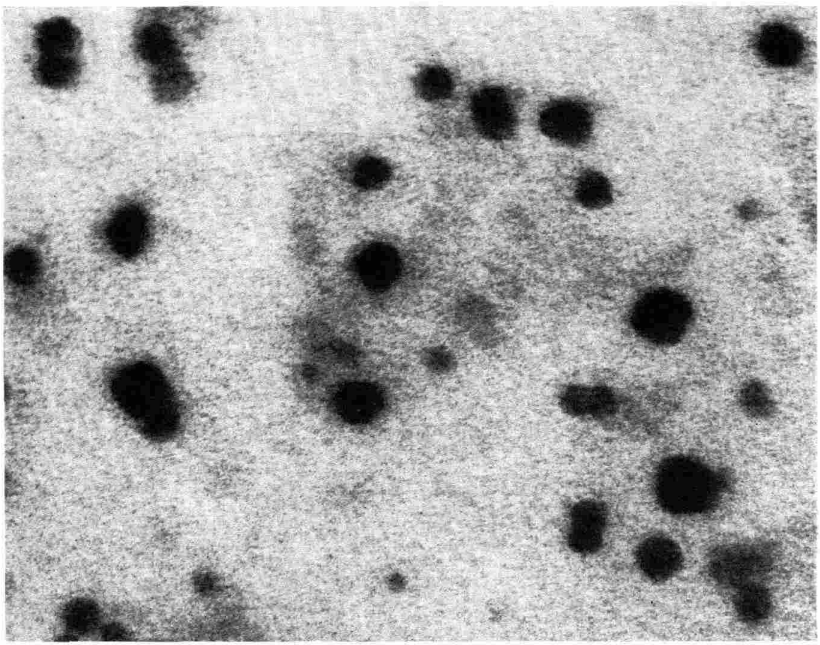

FIG. 3. Electron micrograph of the irradiated opalescent material negatively stained with phosphotungstic acid. Enlarged 61,250 times.

TABLE I

Protein assays of opalescent material

		Protein Wet tissue wt. (mg/gm ± SD)
Control	1	0.144
	2	0.105
	Avg.	0.124 ± 0.023
Irradiated	1	0.200
	2	0.145
	Avg.	0.176 ± 0.028
Difference		+0.052

Each experiment used three animals. The assays for each experiment were performed in triplicate and the standard deviation of these assays was not greater than ±0.005 for any set of assays.

terial antiserum and to the commercial anti-guinea pig serum antiserum. This material was also immunological identical to a serum protein. The immunoelectrophoretic evidence showed the only band forming between serum proteins and anti-opalescent material antiserum was that forming against α_1 and α_2-globulins. The only band forming between the opalescent material and its antiserum appeared in the area of migration of the α_1 and α_2-globulins.

c) *Electron microscopy.* The electron micrograph of the opalescent material, Figure 3, shows a spheroidal, globular material which lacks structure.

d) *Chemical assays.* Data from protein and cholesterol assays are shown in Tables I and II. These data, based on tissue weight, show higher cholesterol and protein content in irradiated epidermal preparations. The contribution of free cholesterol to the total cholesterol content in the samples is shown in Table II. It indicates that the increase in total cholesterol content is due primarily to an increase in free cholesterol.

e) *Thin layer chromotography.* No qualitative difference in phospholipids or neutral lipids were

TABLE II

Free and total cholesterol assays on chloroform-methanol extracts of opalescent material

	Total	Total cholesterol* Wet tissue wt. (mg/gm)	Free cholesterol Wet tissue wt. (mg/gm)
Control	1	0.137	0.061
	2	0.052	0.023
	3	0.092	0.041
	Avg.	0.094	0.042
Irradiated	1	0.131	0.078
	2	0.146	0.085
	3	0.112	0.071
	Avg.	0.130	0.078
Difference		0.036	0.036

Triplicate assays

* Standard deviation of the mean for control mean = 0.018 mg/gm wet tissue weight; for irradiated mean = 0.010 mg/gm wet tissue weight.

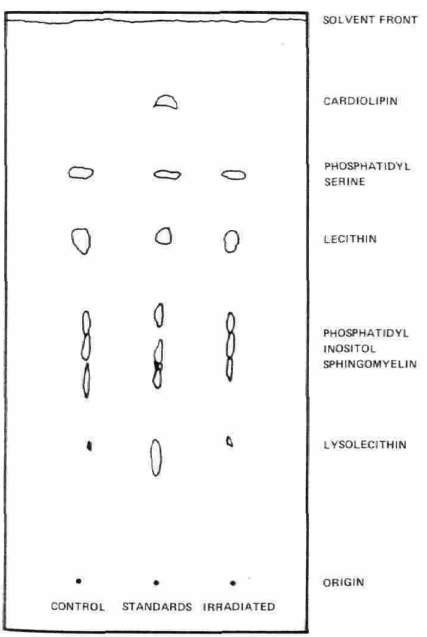

FIG. 4. Tracing of thin layer plate illustrating phospholipids of non-irradiated (control) and irradiated samples.

observed by TLC, as indicated in Figures 4 and 5. Note the absence of cardiolipin in the chromatogram of phospholipids shown in Figure 4. The neutral lipid chromatogram, Figure 5, suggests that there is an increase in fatty acids in the irradiated preparation. However, gas-liquid chromatographic investigations showed the partition of fatty acids to be the same in preparations from irradiated and non-irradiated epidermal preparations.

f) *Solubilization with phospholipase A.* The graph of protein content in fractions eluted from Sephadex G200 filtration of epidermal homogenates incubated with phospholipase A is shown in Figure 6. More protein in the opalescent fractions was released from tissue incubated with this enzyme than was obtained following UV irradiation. The protein was immunologically

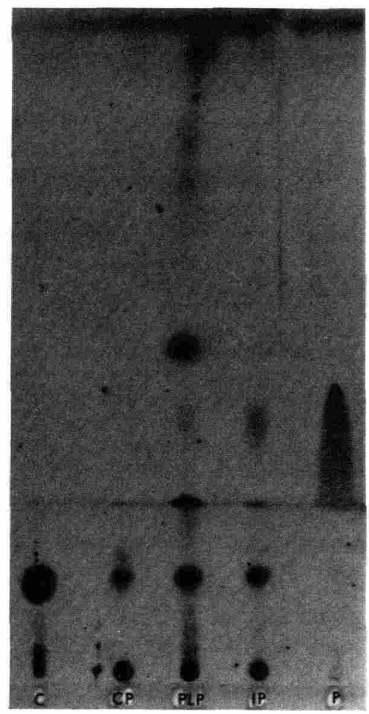

FIG. 5. Thin layers plate showing neutral lipids. Cholesterol (C), non-irradiated opalescent material (CP), phospholipase-treated sample (PLP), irradiated opalescent material (IP), and palmitic acid (P).

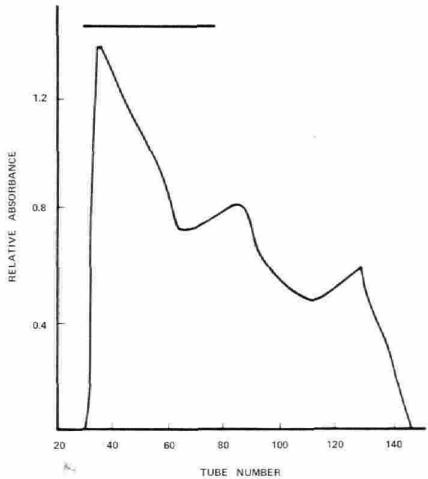

FIG. 6. Graph of protein in fractions from Sephadex column of non-irradiated homogenate (2.93 gm wet weight) treated with phospholipase A. Bar above initial peak denotes opalescent fractions.

identical to material recovered from UV irradiated epidermis. The electrophoresis pattern was also identical to that of opalescent fractions from irradiated epidermis. The neutral lipid pattern is shown in Figure 5 as PLP. There was no difference in the phospholipid pattern with that derived from irradiated epidermis.

DISCUSSION

The evidence presented indicates that ultraviolet light irradiation of guinea pig epidermis results in damage to a particular cellular component. Subsequent homogenization and centrifugation of this tissue results in an opalescent or turbid supernatant solution. The opalescent solution from UV irradiated tissue differs from the solution of non-irradiated tissue by having greater quantities of protein and free cholesterol. The presence of opalescence in control tissue may reflect damage of the cellular component due to procedures of homogenization and centrifugation. A greater amount, however, appears as a result of UV irradiation.

The release or fragmentation of membranes has been accomplished, *in vitro*, by incubation with phospholipase A (13, 14), by sonication (15), or, as suggested by these data, by homogenization of the epidermis in 0.9% sodium chloride. Following these treatments, a large size, opalescent, globular material is released. The product released by the action of phospholipase A on epidermal homogenates was identical with the UV photoproduct when compared by immunological, gel filtration, thin layer chromatographic, and electrophoretic techniques. Rosenberg (15) showed, in his work on the solubilization of the erythrocyte membrane by sonication, that an opalescent product was released. Some properties of this product are similar to the UV photoproduct. Homogenization of epidermal tissue in 0.9% sodium chloride appears to release a similar component from that tissue, since an opalescent solution containing greater amounts of protein are recovered from saline-homogenized samples than from sucrose-homogenized epidermal samples.

There are several considerations in support of the direct effect of UV in membrane damage. First, thermal injury which, *in vivo*, produces vascular changes and a biphasic edema response in guinea pig skin (4), fails to release, *in vitro*, increased amounts of the opalescent material while irradiation with UV *in vitro* does. Second, no qualitative changes of fatty acids, neutral lipids, or phospholipids were apparent in the UV-treated samples. Third, of the chemical assays reported, the only quantitative changes observed were an increase in cholesterol (as free cholesterol) and protein. Fourth, because the response can be provoked *in vitro*, direct participation of serum proteins seems unlikely. Thus the appearance of the opalescent material seems to be a direct response of epidermis to UV irradiation.

Nix, in electron microscopic work on human epidermis (17) and guinea pig epidermis (18), has shown that large membrane-bound vacuoles appear within an hour following exposure to UV and remain for at least 12 hours. These vacuoles contained material which resembled intercellular space material. A similar observation was made by Lane (19) in UV-irradiated cell cultures of KB cells. Nix also observed that besides vacuole-containing cells there were cells without vacuoles which contained diffused cytoplasm of decreased density. Other cells, he noted, contained vacuoles without membranes, which suggests a transitory stage between the diffuse-appearing cells and the membrane-bound vacuoles. Since the quantity of opalescent material in this study is only slightly decreased after a

four hour delay post irradiation, perhaps the vacuoles observed by Nix and by Lane contain this opalescent material.

The opalescent material could thus be the results of UV damage to some cellular component, such as endoplasmia reticulum or Golgi apparatus, causing a decrease in structural organization and release of cholesterol. The evidence does not distinguish whether the material is a cellular organelle or a membrane of a cell organelle. Homogenization of this tissue in a slightly hypertonic medium may result in an aggregation of these lipid-containing fragmented components, resulting in the opalescence of the solution. The absence of cardiolipin suggests that the opalescent material is not derived from mitochondria or lysosomes since these tissues contain cardiolipin (20, 21). The microsomes, with high phospholipid content (22) are, perhaps, better substrate for the phospholipase A than is the plasma membrane which has a lower phospholipid content. The similarity between the enzyme product and the UV photoproduct suggests, therefore, that a microsomal component may be the locus of UV damage. The reorganization of structural elements could result from the accumulation of the damaged material into vacuoles, first by aggregation into membraneless vacuoles, then by incorporation into membrane-bound vacuoles, perhaps to be acted on by lysosomes.

The evidence from this study and from the literature is consistent with the hypothesis that ultraviolet light releases or fragments a cellular component. The damage is observed by electron microscopy as vacuoles. This damage results in the aggregation of lipid-containing cellular components when the tissue is homogenized, and is observed as an opalescent supernatant.

We are indebted to R. E. Nordquist for the preparation of the electron micrograph of the opalescent material.

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